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## A Process to Make Collagen Scaffolds with an Artificial Circulatory System using Rapid Prototyping

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### ABSTRACT

Tissue engineering aims to produce biological substitutes to restore or repair damaged human tissues or organs. The principle strategy behind tissue engineering involves seeding relevant cell(s) onto porous 3D biodegradable scaffolds. The scaffold acts as a temporary substrate where the cells can attach and then proliferate and differentiate. Collagen is the major protein constituent of the extracellular matrix in the human body and therefore an attractive scaffold material. Current collagen scaffolds are foams which limit the mass transport of oxygen and nutrients deep into the scaffold, and consequently cannot support the growth of thick cross-sections of tissue (greater than 500  $\mu\text{m}$ ). We have developed a novel process to make collagen and collagen-hydroxyapatite scaffolds containing an internal artificial circulatory system in the form of branching channels using a sacrificial mould, casting and critical point drying technique. The mould is made using a commercial rapid prototyping system, the Model-Maker II, and is designed to possess a series of connected shafts. The mould is dissolved away and the solvent itself removed by critical point drying with liquid carbon dioxide. Processed hydroxyapatite has been characterised by XRD and FTIR analysis. Tissue engineering with collagen scaffolds possessing controlled internal microarchitecture may be the key to growing thick cross-sections of human tissue.

### INTRODUCTION

Tissue engineering has the potential to significantly improve clinical treatment of damaged human tissue, currently based on organ transplantation and biomaterial implantation, by producing an unlimited supply of immunologically-tolerant 'biological substitutes' that can repair or replace the defect site and grow with the patient. One of the strategies employed involves the expansion of human cells in vitro on biodegradable porous scaffolds. This serves as a substrate for cellular attachment and defines the macroscopic shape of the engineered tissue [1]. Proliferating cells are expected to occupy the freed space created during scaffold degradation and eventually produce a completely natural tissue.

Most scaffolds used for tissue engineering are open-cell foam structures which have resulted in the growth of thin cross-sections of tissue. For example, bone has been grown in vitro to a thickness of 370-500  $\mu\text{m}$  [2, 3]. The small cellular penetration depth of scaffolds may be due to the lack of nutrient and oxygen diffusion deep into the interior of the scaffold; cell colonisation of the scaffold's periphery can become a barrier, or limit, to the diffusion of these essential components. Thus cell migration and survival deep inside the scaffold is hindered.

Rapid prototyping (RP) can impact on tissue engineering by producing scaffolds with an internal artificial circulatory system which can overcome the diffusion constraints of the foam-

structured scaffolds. 3D Printing has been used to prepare poly(glycolic-co-lactic) acid scaffolds with interconnected networks of channels allowing the dynamic culturing of hepatocytes [4]. Polycaprolactone has been extruded using fused deposition modelling and produced honeycomb-like scaffolds used for the culture of bone and cartilage [5]. Similar structures have been manufactured with the 3D Bioplotter<sup>TM</sup> using alginate and fibrin to create hydrogel scaffolds [6]. Hydroxyapatite (HA) scaffolds with a series of radial and orthogonal interconnected channels have been made directly with stereolithography, using HA-loaded photocurable resin [7], or indirectly by casting HA-acrylate suspension into moulds made with stereolithography [8]. However, collagen scaffolds have yet to be made using RP technology.

Collagen is the major protein constituent of the human extracellular matrix and possesses a surface that favours attachment [9], morphology, migration and in certain cases the differentiation of cells [10]. We report a novel process to create collagen scaffolds with an artificial circulatory system which is defined by RP technology. Collagen and collagen-HA composite scaffolds have been manufactured.

## **EXPERIMENTAL DETAILS**

### **Dispersion formulation**

Collagen type I from bovine Achilles tendon (Sigma-Aldrich) was added to 0.05M acetic acid aqueous solution, with pH adjusted to 3.2 by the dropwise addition of 0.5M sodium hydroxide, to yield a 1%w/v concentration. The dispersion was homogenised on ice for 90s using a conventional blender, and then exposed to a vacuum of 1mbar at room temperature for 20min using a vacuum oven (Vacuum Oven, Gallenkamp). The dispersion was stored at 4°C in a refrigerator (ER7610C, Electrolux) until required. HA particles (Captal<sup>®</sup> 60-1, Plasma Biotol) were added to the collagen dispersion and mixed to uniformly distribute the particles.

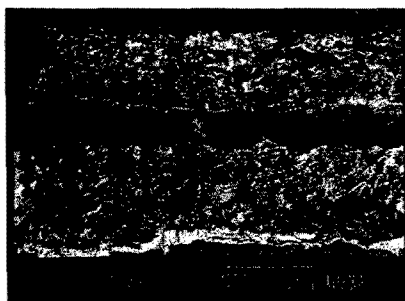
### **The process**

Sacrificial moulds, designed using commercial CAD software, were printed with the Model-Maker II (Solidscape Inc). The proprietary materials, ProtoBuild<sup>TM</sup> and ProtoSupport<sup>TM</sup>, were used to create the mould and support structures, respectively. The support material was removed by immersion in the proprietary solvent, BioAct<sup>TM</sup>, for 30min at 50°C aided by gentle stirring. The moulds were then washed with water and air-dried.

Collagen or collagen-HA dispersion was then cast into the mould and submitted to -20°C in a freezer (EU7120C, Electrolux) for 24h. The samples were then immersed in ethanol for 3h and transferred to a critical point drier (E100, Polaron). There, the ethanol was exchanged with liquid carbon dioxide (CO<sub>2</sub>) at 15-18°C and a pressure of 50bar for 3h before the temperature was increased to 33-36°C, which turned the liquid CO<sub>2</sub> into gaseous CO<sub>2</sub> that was then slowly released from the chamber.

### **Internal scaffold morphology**

Moulds containing frozen collagen and collagen-HA were immersed in liquid nitrogen for several seconds and sectioned with a sharp razor blade to reveal the interior. Samples were then dehydrated in ethanol for 3h and critical point dried for 3h. The sections were then gold-sputter



**Figure 1.** Secondary electron image of processed collagen scaffold with channel running horizontally.

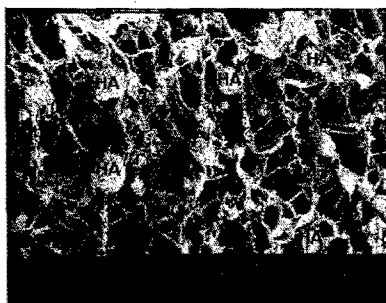
coated (E5400, Biorad, Polaron Division) for 180s. A SEM (JSM-840F, JEOL) operated at 2.5kV was used to image the sectioned surface.

### **HA characterisation**

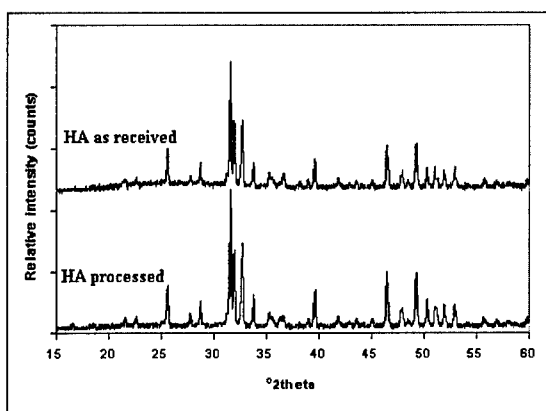
HA powder (Capital<sup>®</sup> 60-1, Plasma Biotol) was immersed in ethanol for 3h and critical point dried for 3h. The powder was analysed before and after processing with X-ray diffraction and Fourier transform infrared spectroscopy. The X-ray diffractometer (PW1710, Philips) was operated at 35kV and a current of 50mA. Samples were scanned from 15-60° (2 $\theta$ ) at a step size of 0.02° (2 $\theta$ ) and time per step of 1s. HA samples for FTIR analysis were mixed with ground potassium bromide powder and pressed into 13mm discs. Samples were analysed in transmission using a FTIR spectrometer (Spectrum 2000 Explorer, Perkin-Elmer) at a resolution of 2cm<sup>-1</sup> and 64 scans.

### **RESULTS**

Figure 1 shows a secondary electron SEM image of a collagen scaffold after processing. The channel running through the full length of the scaffold is approximately 400 $\mu$ m in width.

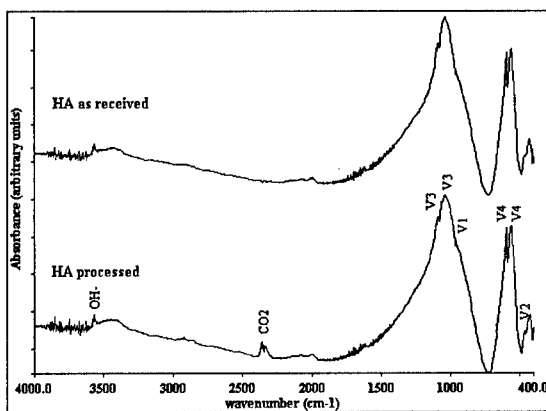


**Figure 2.** Secondary electron image of scaffold showing interconnecting porosity of collagen matrix and hydroxyapatite (HA) particles.



**Figure 3.** XRD spectra of hydroxyapatite as received and after processing. The same HA peaks are evident in both spectra.

The collagen is organised into an open-cell pore structure as shown in figure 2. Furthermore, HA particles added to the collagen dispersion are also present after processing and randomly distributed throughout the scaffold. XRD analysis of processed HA particles exhibited no change in the spectrum compared to the material as received from the manufacturer as shown in figure 3. Furthermore, FTIR analysis of processed HA particles revealed the phosphate  $\nu_1$ ,  $\nu_2$ ,  $\nu_3$ ,  $\nu_4$  and hydroxyl stretching peaks of HA are present and at their characteristic wavenumbers as shown in figure 4. A  $\text{CO}_2$  peak at  $2300\text{--}2400\text{cm}^{-1}$ , absent in the spectrum of HA as received, is evident in the spectrum of processed HA.



**Figure 4.** FTIR of HA as received and after processing. The characteristic phosphate  $\nu_1$ ,  $\nu_2$ ,  $\nu_3$ ,  $\nu_4$  peaks of HA are labelled accordingly. Processed HA reveals a  $\text{CO}_2$  peak at  $2300\text{--}2400\text{cm}^{-1}$ .

## DISCUSSION

The process, presented in this paper, is able to create collagen scaffolds with controlled internal channels as shown in figure 1. The channels are intended to act as an artificial circulatory system by permitting the flow of nutrient-rich medium and supporting the migration and survival of cells deep into the scaffold. The channels have smaller dimensions compared to the mould shafts used to define them [11]. This shrinkage of the collagen matrix is due to critical point drying with liquid CO<sub>2</sub> [11, 12].

Processing does not effect the tertiary structure of collagen [11]. Therefore, processed collagen should retain its natural functions, such as cell attachment properties, as these are highly dependent on protein structure. This however remains to be examined.

Freezing the aqueous component of the collagen dispersion results in the formation of ice crystals that aggregate the collagen into the interstitial spaces to create an open-cell pore structure [14] as shown in figure 2. However, the mould and ice crystals need to be removed to expose the internal structures and this is achieved by dissolving in ethanol. Collagen is insoluble in ethanol. The ethanol solvent need to be removed in order to crosslink, rehydrate and prepare the scaffold for cell culturing. Critical point drying with liquid CO<sub>2</sub> serves such a purpose.

For bone tissue engineering, HA particles can be added to the collagen dispersion, as these are the components that make up the natural extracellular matrix of bone. Figure 2 shows such a composite scaffold. The collagen and HA should provide an environment familiar and favourable for the growth of osteogenic cells. Processing does not alter the HA phase. XRD analysis did not detect any phase change in HA after processing as shown in figure 3. Furthermore, FTIR analysis revealed no evidence of changes in the characteristic peaks assigned to HA as shown in figure 4. However, a new peak attributed to CO<sub>2</sub> was revealed after processing. This may be due to the CO<sub>2</sub> used for critical point drying or environmental CO<sub>2</sub> present while acquiring the spectrum.

The greatest challenge facing the technology is whether residual mould material is present on the collagen scaffold after processing. This is currently being investigated using electron probe microanalysis. Research efforts are also aimed at printing a biocompatible mould material, which would eliminate any fears of possible mould residues. The biological performance of these processed collagen and collagen-HA scaffolds still remains to be examined.

## CONCLUSIONS

We have developed a process to make collagen or collagen-hydroxyapatite scaffolds with an artificial circulatory system. Moulds made using rapid prototyping technology define the internal and external morphology of the scaffolds. Mould removal is achieved by chemical dissolution and the solvent is removed by critical point drying with liquid carbon dioxide. Channels with predefined dimensions and shape are incorporated into the open-celled collagen scaffold. Composite scaffolds comprising of collagen and hydroxyapatite can also be produced. The process does not alter the phase of hydroxyapatite particles.

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